Determination of nucleotides in Chinese human milk by high-performance liquid chromatography–tandem mass spectrometry

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ORIGINAL PAPER

Determination of nucleotides in Chinese human milk by high-performance liquid chromatography–tandem mass spectrometry

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Abstract The determination of nucleotide profile in human milk is an important issue for making nucleotide-fortified dairy products. Here, a method for the simultaneous quantitation of five monophosphate nucleotides (cytidine 5′-monophosphate (CMP), uridine 5′-monophosphate (UMP), adenosine 5′-monophosphate (AMP), inosine 5′-monophosphate (IMP), guanosine 5′-monophosphate (GMP)) in milk using high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) was developed. The nucleotides were separated using a C_{18} column and gradient elution with water containing 0.1% (*v*/*v*) formic acid and acetonitrile. The method was validated with spike recoveries of 90.3–102.7% and 81.2–96.2%, and repeatability relative standard deviations of 2.5–4.3% and 2.6–4.6% for fresh bovine milk and milk powder, respectively. The method was applied for the assay of Chinese human milk samples collected from 59 nursing mothers with 2–4 months of lactation in Zhejiang, China. The mean (±SD) contents of CMP, UMP, AMP, IMP, and GMP are 3.1±1.8, 2.2 ±2.2, 0.23±0.18, 0.018±0.023, and 0.24±0.19 $mg.kg^{-1}$, respectively. The results of this work may serve as a guideline for the humanizing bovine milk-based infant formulas by supplementing the five monophosphate nucleotides for Chinese infants.

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1 Introduction

Human milk is the nonpareil food for infants during the first 6 months of life. It contains a large number of nutrients which cover all the newborn's requirements (German et al. 2002). As one of the main components of human milk, protein and nonprotein nitrogen plays an important role in human health. The nonprotein nitrogen accounts for approximately 20–25% of the total nitrogen content, and nucleotides have been found to account for 2–5% of the nonprotein nitrogen in human milk (Janas and Picciano 1982). Nucleotides have been demonstrated to play key roles in efficient immune system function, enhanced metabolism of fatty acids, and improved gastrointestinal tract repair after damage (Carver and Walker 1995; Schlimme et al. 2000).

In bovine milk, only 2–5% of the total nitrogen is nonprotein nitrogen, and the total nucleotide content in bovine milk is much lower than that in human milk (Gil and Sanchez-Medina 1982; Ferreira 2003). Therefore, fortification of nucleotides to bovine milk-based infant formulas would be beneficial for infant health. It is an important issue to establish a reliable analytical method for the simultaneous determination of nucleotides in milks, infant formulas, and food matrices for the purposes of food safety, nutritional database information, regulatory compliance, and quality control.

The most commonly used technique for nucleotide analysis is high-performance liquid chromatography (HPLC) based on different column separation modes and different detection systems. The separation is usually performed by reverse-phase liquid chromatography (RPLC) (Gill and Indyk 2007; Ren et al. 2011; Studzińska and Buszewski 2013), ion-pair RPLC (Viñas et al. 2010; Yang et al. 2010), ion exchange chromatography (Studzińska et al. 2014; Viñas et al. 2009), and hydrophilic interaction liquid chromatography (García-Gómez et al. 2013). The detection is usually achieved by UV or mass spectrometry (Gill et al. 2013; Neubauer et al. 2012). Good resolutions of nucleotides are required when using UV detector, which can be achieved by RPLC and ion-pair RPLC. Gill and Indyk (2007) reported excellent resolution of five nucleotides by RPLC through gradient elution with KH_2PO_4 solutions in 60 min, but it is time-consuming. Ion-pair RPLC is commonly used to separate nucleotides with excellent resolution, and phosphate buffer is used as the mobile phase. The disadvantage is the risk of crystallization of the system and the longer column equilibration time. The nonvolatile phosphate salt or formate buffer and the ion-pair reagent (Viñas et al. 2010) will bring about ion suppression in the electrospray ionization and contaminate the ion source of the mass analyzer. The tandem MS detection is much more specific than UV or MS detection method. Accurate quantification can be achieved even though the resolution is not very good. Methods for the analysis of nucleotides other than liquid chromatography include capillary electrophoresis (CE) (Ding et al. 2011), capillary electrophoresis–inductively coupled plasma mass spectrometry (Yeh and Jiang 2002), and capillary electrochromatography (Chen et al. 2010).

The aim of this work was to assay the nucleotide composition of Chinese human milk using liquid chromatography coupled to tandem mass spectrometry. The method developed in this work employed a simple binary gradient elution protocol with a C_{18} column to separate the nucleotides and tandem mass spectrometry to detect and quantify the nucleotides with high selectivity and accuracy. Only highly MS-compatible solvents of water, formic acid, and acetonitrile are used as eluents, which is beneficial for MS detection by avoiding the use of ion-pair reagents and phosphate buffer. The described technique has been applied to the analysis of 59 Chinese human milk samples. The information provided in this work will help add to the body of knowledge on infant nucleotide intake when human milk is the main diet. The resulting data will serve to improve commercial infant formulas and bring them closer to the reference standard represented by human milk when making infant formulas using bovine milk as raw material.

2 Materials and methods

2.1 Materials

Cytidine 5′-monophosphate (CMP) disodium salt, uridine 5′-monophosphate (UMP) disodium salt, adenosine 5′-monophosphate (AMP) sodium salt, guanosine 5′-monophosphate (GMP) disodium salt, and inosine 5′-monophosphate (IMP) disodium salt were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade methanol and acetonitrile were purchased from Fisher Scientific (Shanghai, China). Formic acid and *n*-hexane were of analytical grade.

2.2 Milk sample preparation

Fifty-nine human milk samples were individually collected from lactating mothers at 2–4 months postpartum in Ningbo and Hangzhou, Zhejiang, China. After collection, samples were immediately frozen and kept at −20 °C until analysis. Fresh bovine milk and milk powder were kindly provided by Hangzhou Shuangfeng Dairy Products Co., Ltd. and Zhejiang Beingmate Group Co. Ltd., respectively.

Prior to analysis, the human milk sample was allowed to thaw to room temperature and 5.0 mL was transferred to a 50-mL polypropylene centrifuge tube. To this was added 20 mL of *n*-hexane and the tube was capped and shaken sufficiently to ensure complete mixing. The sample was then centrifuged at 6,000 rpm for 15 min. The fat was dissolved in the *n*-hexane layer and it was discarded. Proteins and water insoluble impurities were precipitated in the bottom and discarded. The nucleotides, carbohydrates, and oligopeptides were dissolved in the water layer. An aliquot of the aqueous solution was further passed through a membrane (5,000 $g.mol^{-1}$ cutoff) to remove solutes with high molecular weight (>5,000 $g.mol^{-1}$). The nucleotides in the filtrate were determined by the HPLC–MS/MS method.

One gram of milk powder was dissolved in 8 mL of deionized water. The fresh bovine milk and milk powder reconstituted samples were prepared at the same way as described above for human milk sample. The densities of fresh bovine milk and human milk were both 1.02 $g.mL^{-1}$ when converting from sample volume to sample mass.

2.3 Instrumentation

The HPLC system used in this work consisted of G1312A series 1200 binary pump, G1367B autosampler, and G1316A column compartment (Agilent Technologies, USA). Chromatographic separation was achieved using an XDB-C_{18} column (4.6 mm×50 mm, 1.8 μm) obtained from Waters (Milford, MA, USA). The column was maintained at 50 °C and gradiently eluted with water containing 0.1% (*v*/*v*) formic acid (eluent A) and acetonitrile (eluent B) at a flow rate of 0.3 mL.min^{-1}. The mobile phase composition was held constant at 1% B for 7 min, then increased to 90% B within 0.5 min and held for 6 min. Subsequent reconstitution of the starting conditions within 0.5 min and re-equilibration with 1% B for 6 min resulted in a total analysis time of 20 min. The injection volume was 5 μL using an autosampler. The HPLC system was coupled to the mass spectrometer.

The MS/MS system consisted of a triple quadrupole mass spectrometer with an ion source equipped with an electrospray ionization (ESI) probe (Agilent 6410B). The mass spectrometer was operated in ESI$^-$ mode using nitrogen as the collision and drying gas. The instrumental parameters were set as follows: curtain gas at 40 psi, ion spray voltage at 4.0 kV, desolvation temperature at 350 °C, and desolvation gas flow rate at 10 L.min^{-1}. The quantitation was performed in multiple-reaction monitoring (MRM) mode. Instrument settings and MRM transitions for the generation of product ions for nucleotides are given in Table 1.

In this work, the standard additions and external standardization techniques using unlabelled standards were used for calibration. The detailed calibration method includes the following: (1) each of the samples was split into two parts and one part was spiked with known amount of nucleotide (m_a), and the peak areas of both parts were determined by HPLC–MS/MS; (2) the difference of peak areas between the spiked part (A_s) and the original part (A) is related to the amount spiked (m_a); (3) the nucleotide content in the sample (m) can be obtained by comparing the peak area of the sample (A) and the peak area difference between both parts (A_s–A); and (4) the peak area (A_e) of external standardization method at m_a was also determined, and the method accuracy (recovery) was evaluated by comparing (A_s–A) with A_e.

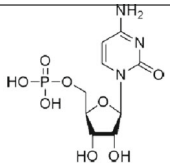
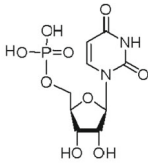
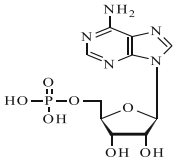
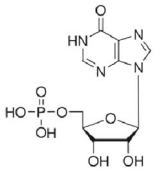
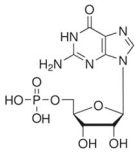
3 Results

3.1 HPLC–MS/MS method

Appropriate standards were used to establish retention times of each nucleotide and the HPLC separation was optimized by adjusting the mobile phase composition and the flow rate. The optimized method allowed a fast and effective separation of five nucleotides within 10 min at a flow rate of 0.3 mL.min^{-1} as illustrated in Fig. 1. The resolution of IMP and GMP using a simple binary elution protocol with a C_{18} column is inadequate. Thus, the quantitation using conventional LC detection method, such as UV detector, is not accurate for this protocol.

The tandem mass spectrometry offers advantages over conventional LC detection method, and accurate quantitation can be achieved via MS/MS when the chromatographic resolution is poor. The five peaks separated by the described liquid

Table 1 MS/MS parameters for the five monophosphate nucleotides

Nucleotide	Chemical structure	Precursor ion (m/z)	Product ion (m/z)	Collision energy (V)	Cone voltage (V)	Retention time (min)
CMP		322	211 139 97	18 18 18	135	4.2
UMP		323	211 139 97	20 20 20	135	4.8
AMP		346	211 134 97	20 20 20	135	5.8
IMP		347	194 151 97	18 18 18	135	6.4
GMP		362	211 150 97	20 20 20	135	6.7

CMP cytidine 5′-monophosphate, *UMP* uridine 5′-monophosphate, *AMP* adenosine 5′-monophosphate, *GMP* guanosine 5′-monophosphate, *IMP* inosine 5′-monophosphate

chromatographic protocol were determined by ESI-MS/MS and the MS/MS spectra of five nucleotides are shown in Fig. 2. Peaks 1 to 5 correspond to CMP, UMP, AMP, IMP, and GMP, respectively. Accurate quantitation of the five nucleotides can be achieved even though the resolution of chromatographic separation is not excellent, which is attributed to the high degree of selectivity afforded by MRM of tandem MS as illustrated in Fig. 1. Therefore, the HPLC–MS/MS allowed us to simultaneously detect all five nucleotides in milk samples.

3.2 Method validation

3.2.1 Linear range and detection limits

Under the optimized HPLC–MS/MS conditions for the separation and detection of nucleotides, the method was validated by determining linearity, limit of detection

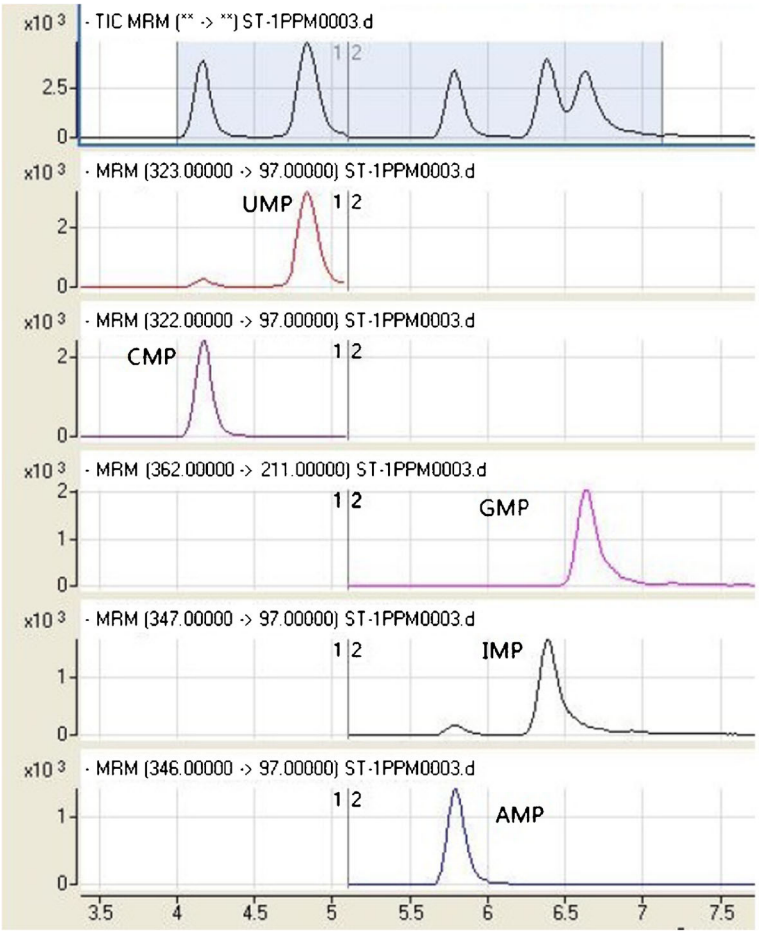


Fig. 1 MRM chromatograms of the five nucleotide standards by HPLC–MS/MS

(LOD), precision, and recovery. Calibration curves were obtained according to the external standard method by least-squares linear regression analysis of the peak area versus the nucleotide concentration of multilevel standards. Good linearity was obtained for all five nucleotides with high correlation coefficients (R^2>0.991). The LOD was defined as three times the signal-to-noise ratio. The detection limits were determined by serial dilution of sample solutions using the established HPLC–MS/MS method until the signal-to-noise at 3:1. These values are also given in Table 2.

3.2.2 Recovery and precision

The method accuracy was confirmed by evaluating recovery using the standard additions method. Fresh bovine milk and milk powder samples were used and spiked at three different known amounts of nucleotides (0.05, 0.5, and 5 μg.kg^{-1} sample) to investigate the precision and accuracy of this analytical method. Intra-run precision, as repeatability, was determined using six replicate analyses of a sample of fresh bovine

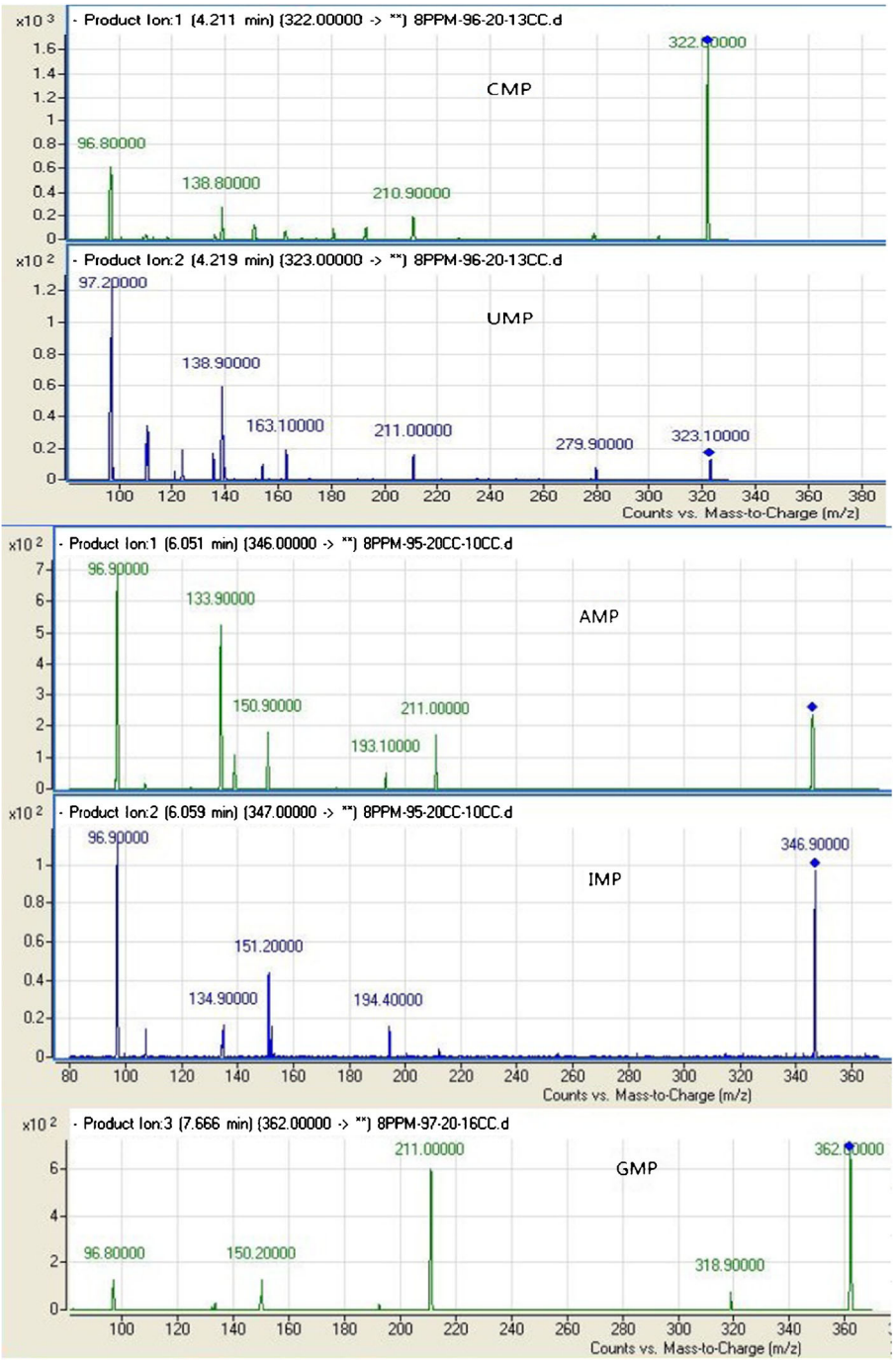


Fig. 2 MS/MS spectra for the five monophosphate nucleotides

milk or milk powder spiked with known concentrations of corresponding nucleotides. The percentage recoveries of added nucleotides were then calculated after accounting

Table 2 Method performance as linearity and detection limit for the determination of five monophosphate nucleotides by HPLC–MS/MS

Nucleotide	Linear range ($mg.kg^{-1}$)	Detection limit ($mg.kg^{-1}$)	R^2
CMP	0.03–10	0.010	0.993
UMP	0.03–10	0.010	0.992
AMP	0.05–10	0.015	0.994
IMP	0.05–10	0.015	0.993
GMP	0.05–10	0.015	0.991

CMP cytidine 5′-monophosphate, *UMP* uridine 5′-monophosphate, *AMP* adenosine 5′-monophosphate, *GMP* guanosine 5′-monophosphate, *IMP* inosine 5′-monophosphate

for the endogenous nucleotides contribution. The accuracy was calculated by comparing the spiked concentrations with the actual measured concentrations of the samples spiked with all five nucleotides. The precision was assessed by determining the relative standard deviation (RSD). The results are presented in Table 3.

The recoveries of the nucleotides from spiked fresh bovine milk and milk powder samples varied within the range of 90.3–102.7% and 81.2–96.2%, respectively. The RSD for fresh bovine milk samples and milk powder samples were 2.5–4.3% and 2.6–4.6%, respectively. These values confirmed that the precision of the method was satisfactory for the analysis of the five nucleotides.

Table 3 Average recovery tests and precision of the nucleotides (data are the mean of six separate experiments)

Nucleotide	Amount spiked ($\mu g.kg^{-1}$)	Fresh bovine milk		Milk powder	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
CMP	0.05	92.7	4.1	81.8	3.5
	0.5	99.4	2.5	91.9	2.9
	5.0	102.7	3.0	93.7	3.4
UMP	0.05	90.3	3.3	86.4	4.3
	0.5	91.3	2.6	96.2	3.0
	5.0	90.9	3.2	92.4	2.9
AMP	0.05	95.7	3.7	81.2	4.6
	0.5	90.8	3.1	87.5	3.7
	5.0	91.7	2.8	89.8	2.7
IMP	0.05	98.1	4.3	89.5	4.0
	0.5	93.1	3.3	92.0	2.6
	5.0	94.6	2.6	93.2	2.6
GMP	0.05	93.2	3.6	82.9	4.5
	0.5	96.7	2.7	89.6	3.4
	5.0	91.1	3.1	92.3	2.8

CMP cytidine 5′-monophosphate, *UMP* uridine 5′-monophosphate, *AMP* adenosine 5′-monophosphate, *GMP* guanosine 5′-monophosphate, *IMP* inosine 5′-monophosphate

Table 4 Mean nucleotide contents of 59 Chinese human milk samples (2–4 months of lactation) collected from Ningbo and Hangzhou, Zhejiang, China

Nucleotide	Range ($mg.kg^{-1}$)	Mean±SD ($mg.kg^{-1}$)
CMP	0.33–7.4	3.1±1.8
UMP	0.26–15	2.2±2.2
AMP	0.022–0.85	0.23±0.18
IMP	0–0.14	0.018±0.023
GMP	0–0.95	0.24±0.19
Total 5 nucleotides	0.82–24	5.8±3.9

CMP cytidine 5′-monophosphate, *UMP* uridine 5′-monophosphate, *AMP* adenosine 5′-monophosphate, *GMP* guanosine 5′-monophosphate, *IMP* inosine 5′-monophosphate

3.3 Analysis of Chinese human milk samples

The content ranges, mean values, and standard deviations (SD) for each of the nucleotides and the total five nucleotides are summarized in Table 4. Significant differences in contents of each of the nucleotides and the total five nucleotides between individuals were noted. It was observed that all 59 Chinese human milk samples contained relatively large quantities of CMP and UMP. CMP and UMP measured in the present study comprised the first and second largest fractions of the total nucleotide content, respectively. AMP and GMP are present at similar levels but at significantly lower amounts. The content of IMP is lowest among the five nucleotides and IMP was undetected in 20 of the 59 human milk samples analyzed. The total concentration of five free nucleotides varied significantly from 0.82–24 $mg.kg^{-1}$. Overall, CMP, UMP, AMP, GMP, and IMP in the Chinese human milk measured in this work account for 53.5, 38.2, 3.87, 4.16, and 0.31% of the total five monophosphate nucleotides, respectively.

4 Discussion

The content of nucleotides in human milk is varied with diet, country, and stage of lactation. It has been reported that levels of nucleotide declined as the lactation period progressed (Janas and Picciano 1982; Leach et al. 1995; Tressler et al. 2003). In this study, CMP and UMP comprised the first and second largest fractions of the total nucleotide, and IMP constituted only a very small percentage of nucleotides. This relative proportion is consistent with previous reports for American, European (Leach et al. 1995), and Asian women (Tressler et al. 2003). The mean content of CMP in the present study is very close to the value of 3.2 $mg.L^{-1}$ for American women at 12 weeks of lactation (Janas and Picciano 1982) but much lower than the value of 21 $mg.L^{-1}$ for 14 Swedish mothers at 3–24 weeks of lactation (Thorell et al. 1996) and the value of 16 $mg.L^{-1}$ in Taiwanese human milk when regardless of the stage of lactation for 24 individual milk samples (Liao et al. 2011). The mean content of UMP in this study is basically at the same level as previously reported values of 1.4 $mg.L^{-1}$ for American women at 12 weeks of lactation (Janas and Picciano 1982), 3.6 $mg.L^{-1}$ for 14 Swedish

mothers at 3–24 weeks of lactation (Thorell et al. 1996), and 1.8 mg.L^{-1} in Taiwanese human milk when regardless of the stage of lactation for 24 individual milk samples (Liao et al. 2011).

The mean contents of AMP and GMP in this study are basically at the same levels as previously reported values (Liao et al. 2011; Janas and Picciano 1982; Thorell et al. 1996). The IMP concentration measured in this study is very low; IMP was undetected in 20 of the 59 human milk samples and the maximum level is 0.14 mg.kg^{-1}. Our data are consistent with the previously reported results (Leach et al. 1995; Thorell et al. 1996; Tressler et al. 2003). IMP was detectable in colostrum (1 to 3 days postpartum) samples at very low level (mean value of 1.2 mg.L^{-1}) but was below the limit of detection (0.25 mg.L^{-1}) in other milk as lactation period progressed (Leach et al. 1995; Tressler et al. 2003). Thorell et al. (1996) reported that IMP was not detected in the milk samples of 14 Swedish mothers at 3–24 weeks of lactation. Opposite results were reported to be 2.9 mg.L^{-1} for American women at 12 weeks of lactation (Janas and Picciano 1982) and 8.8 mg.L^{-1} in Taiwanese human milk when regardless of the stage of lactation for 24 individual milk samples (Liao et al. 2011). The mean level of total five nucleotides in Chinese women of this work is 5.8±3.9 mg.kg^{-1}, which is at similar level with American women at 12 weeks of lactation (Janas and Picciano 1982) but lower than other previous reports (Liao et al. 2011; Janas and Picciano 1982; Thorell et al. 1996).

Only five nucleotides in the form of monophosphate salts are used for the production of nucleotide-enriched infant formulas (Cosgrove 1998). Nucleotide-supplemented formulas generally contain between 20 and 70 mg.kg^{-1} when mixing 1 g of milk powder with 8 g of water (Bacha et al. 2013; Carver 2003; Ren et al. 2011), which is within the content range of the total potentially available nucleosides (TPAN) but higher than the free nucleotide content in the human milk. The mean TPAN concentration in human milk was reported to be about 70 mg.L^{-1} for Asian, European, and American women, and the free nucleotides generally account for 30–40% of TPAN (Leach et al. 1995; Liao et al. 2011; Tressler et al. 2003). It is essential to determine TPAN of Chinese human milk for better understanding the nucleotide profiles, which is underway in our lab.

5 Conclusions

An analytical method based on HPLC coupled to MS/MS was established for the reliable, rapid, and routine determination of five monophosphate nucleotides in dairy products. The sample pretreatment was simple and time-saving, which is beneficial for routine analysis. The obtained results update the human milk database for Chinese people and provide new information about human milk nucleotides from the breastfeeding mothers in Zhejiang, China. The results of the present study may serve as a guideline for the humanizing bovine milk-based infant formulas by supplementing the five monophosphate nucleotides for Chinese infants.

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Conflict of interest Gongnian Xiao, Hailong Xiao, Yinbang Zhu, and Yuru You declare that they have no conflicts of interest.

Compliance with ethics guideline This article does not contain any studies with human or animal subjects performed by any of the authors.

References

Bacha U, Nasir M, Ali MA, Muhammad J, Sheikh AA (2013) Nucleotides supplementation improves various function of the body. J Anim Health Prod 1:1–5

Carver JD (2003) Advances in nutritional modifications of infant formulas. Am J Clin Nutr 77(suppl):1500S–1554S

Carver JD, Walker WA (1995) The role of nucleotides in human nutrition. J Nutr Biochem 6:58–72

Chen X, Yang F, Wang Y, Li S (2010) CE and CEC of nucleosides and nucleotides in food materials. Electrophoresis 31:2092–2105

Cosgrove M (1998) Perinatal and infant nutrition: nucleotides. Nutrition 14:748–751

Ding X, Li Y, Song B, Zhao S, Wang Z (2011) Determination of 5′-mononucleotides in infant formula by capillary electrophoresis with ultraviolet detection. Dairy Sci Technol 91:759–770

Ferreira I (2003) Quantification of non-protein nitrogen components of infant formulae and follow-up milks: comparison with cows and human milk. Br J Nutr 90:127–133

García-Gómez D, Rodríguez-Gonzalo E, Carabias-Martínez R (2013) Stationary phases for separation of nucleosides and nucleotides by hydrophilic interaction liquid chromatography. TrAC 47:111–128

German JB, Dillard CJ, Ward RE (2002) Bioactive components in milk. Curr Opin Clin Nutr Metab Care 5: 653–658

Gil A, Sanchez-Medina F (1982) Acid-soluble nucleotides of human milk at different stages of lactation. J Dairy Res 49:301–307

Gill BD, Indyk HE (2007) Development and application of a liquid chromatographic method for analysis of nucleotides and nucleosides in milk and infant formulas. Int Dairy J 17:596–605

Gill BD, Indyk HE, Manley-Harris M (2013) Analysis of nucleosides and nucleotides in infant formula by liquid chromatography–tandem mass spectrometry. Anal Bioanal Chem 405:5311–5319

Janas LM, Picciano MF (1982) The nucleotides profile of human milk. Pediatr Res 16:659–662

Leach JL, Baxter JH, Molitor BE et al (1995) Total potentially available nucleosides of human milk by stage of lactation. Am J Clin Nutr 61:1224–1230

Liao KY, Wu TC, Huang CF, Lin CC, Huang IF, Wu L (2011) Profile of nucleotides and nucleosides in Taiwanese human milk. Pediatr Neonatol 52:93–97

Neubauer S, Rugova A, Chu DB, Drexler H, Ganner A, Sauer M, Mattanovich D, Hann S, Koellensperger G (2012) Mass spectrometry based analysis of nucleotides, nucleosides, and nucleobases–application to feed supplements. Anal Bioanal Chem 404:799–808

Ren Y, Zhang J, Song X, Chen X, Li D (2011) Simultaneously determination of 5'-monophosphate nucleotides in infant formulas by HPLC-MS. J Chromatogr Sci 49:332–337

Schlimme E, Martin D, Meisel H (2000) Nucleosides and nucleotides: natural bioactive substances in milk and colostrum. Br J Nutr 84(Suppl 1):S59–S68

Studzińska S, Buszewski B (2013) Effect of mobile phase pH on the retention of nucleotides on different stationary phases for high-performance liquid chromatography. Anal Bioanal Chem 405:1663–1672

Studzińska S, Rola R, Buszewski B (2014) Determination of nucleotides in infant milk formulas using novel dendrimer ion-exchangers. J Chromatogr B 949–950:87–93

Thorell L, Sjöberg L, Hernell O (1996) Nucleotides in human milk: sources and metabolism by the newborn infant. Pediatr Res 40:845–852

Tressler RL, Ramstack MB, White NR, Molitor BE, Chen NR, Alarcon P, Masor ML (2003) Determination of total potentially available nucleosides in human milk from Asian women. Nutrition 19:16–20

Viñas P, Campillo N, López-García I, Martínez-López S, Vasallo I, Hernández-Córdoba M (2009) Anion exchange liquid chromatography for the determination of nucleotides in baby and/or functional foods. J Agric Food Chem 57:7245–7249

Viñas P, Campillo N, Melgarejo GF, Vasallo M, López-García I, Hernández-Córdoba M (2010) Ion-pair high-performance liquid chromatography with diode array detection coupled to dual electrospray atmospheric

pressure chemical ionization time-of-flight mass spectrometry for the determination of nucleotides in baby foods. J Chromatogr A 1217:5197–5203

Yang FQ, Li DQ, Feng K, Hu DJ, Li SP (2010) Determination of nucleotides, nucleosides and their transformation products in *Cordyceps* by ion-pairing reversed-phase liquid chromatography–mass spectrometry. J Chromatogr A 1217:5501–5510

Yeh C, Jiang S (2002) Determination of monophosphate nucleotides by capillary electrophoresis inductively coupled plasma mass spectrometry. Analyst 127:1324–1327